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Relaxin Induces Matrix-Metalloproteinases-9 and -13 via RXFP1: Induction of MMP-9 Involves the PI3K, ERK, Akt and PKC-ζ Pathways

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Abstract

We determined the precise role of Relaxin Family Peptide (RXFP) receptors-1 and -2 in the regulation of MMP-9 and -13 by relaxin, and delineated the signaling cascade that contributes to relaxin's modulation of MMP-9 in fibrocartilaginous cells. Relaxin treatment of cells in which RXFP1 was silenced resulted in diminished induction of MMP-9 and -13 by relaxin, whereas overexpression of RXFP1 potentiated the relaxin-induced expression of these proteinases. Suppression or overexpression of RXFP2 resulted in no changes in the relaxin-induced MMP-9 and -13. Studies using chemical inhibitors and siRNAs to signaling molecules showed that PI3K, Akt, ERK and PKC-ζ and the transcription factors Elk-1, c-fos and to a lesser extent NF-κB are involved in relaxin's induction of MMP-9. Our findings provide the first characterization of signaling cascade involved in the regulation of any MMP by relaxin and offer mechanistic insights on how relaxin likely mediates extracellular matrix turnover.

Keywords

Relaxin receptors; synovial joint fibrochondrocytes; matrix metalloproteinases; ERK; Akt; PKC-ζ; c-fos; Elk-1; NF-κB; transcription factors

1. Introduction

Relaxin H2, the major stored and circulating form of relaxin in humans, is a 6-kDa polypeptide belonging to the insulin family of structurally related hormones, whose activities are distinct from other members of this family (James et al., 1977; Schwabe and McDonald, 1977). It is primarily synthesized in the corpus luteum and placenta, and is involved in multiple and diverse physiologic functions including extracellular matrix (ECM) remodeling, neoangiogenesis, and vasodilation (Bani, 1997; Bathgate et al., 2003; Ivell and Bathgate, 2006; Schwabe and Bullesbach, 2007; Sherwood, 2004). Of these, the most important physiologic function of relaxin appears to be the remodeling of ECM in reproductive and non-reproductive tissues. In these target tissues, relaxin is known to decrease collagen and glycosaminoglycan content including that of small proteoglycans, decorin and biglycan that contribute to collagen fibril organization and formation (Downing

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and Sherwood, 2011; Unemori et al., 1992). In most reproductive tissues, including the cervix, uterus, ovary, breast, deciduas and the fibrocartilaginous pubic symphysis, relaxin mediates changes in matrix composition and organization by modulating the synthesis of matrix macromolecules (Hwang et al., 1996) or altering the expression of matrix-degrading enzymes (Mushayandebvu and Rajabi, 1995) or both (Hwang et al., 1996; Mushayandebvu and Rajabi, 1995). Further evidence of the role of relaxin in ECM turnover is provided by observations in relaxin knockout mice that demonstrate accumulation of collagen in the nipple and diminished relaxation of the pubic symphysis during parturition (Zhao et al., 1999), as well as progressive fibrosis of the lungs (Samuel et al., 2005; Zhao et al., 1999).

Besides its modulation of matrix turnover in reproductive tissues, relaxin regulates tissue turnover in lung and alveolar fibroblasts, renal and vascular tissues (Lekgabe et al., 2005; McGuane and Parry, 2005; Unemori and Amento, 1990; Unemori et al., 1996), and in synovial joint fibrocartilage (Kapila, 2003; Naqvi et al., 2005). In both the synovial joint and pubic symphysis fibocartilaginous tissues, the relaxin-mediated alterations in matrix composition appear to result largely due to increased degradative responses rather than due to changes in matrix synthesis (Naqvi et al., 2005; Samuel et al., 1996; Samuel et al., 1998). More specifically, relaxin enhances degradation of these tissues by upregulating specific members of the matrix metalloproteinase (MMP) family of enzymes, namely MMP-1 (collagenase-1), -3 (stromelysin-1), -9 (92 kDa gelatinase) and -13 (collagenase-3) (Kapila and Xie, 1998; Kapila, et al., 2009; Naqvi et al., 2005). MMPs are a family of up to 25 enzymes that are characterized by their ECM substrate specificity, zinc-dependent activity, inhibition by tissue inhibitors of metalloproteinase, secretion as a zymogen and sequence similarities (Kessenbrock et al., 2010; Morrison et al., 2009). Between them, MMPs can degrade all the major matrix macromolecules of connective tissues including collagen, fibronectin and proteoglycans as well as many minor proteins.

The net matrix remodeling activities of relaxin within target tissues are likely determined by the levels of its receptors and the subsequent signaling initiated by activation of the receptors by the binding with the hormone ligand. Relaxin is known to bind to and activate the leucine-rich guanine nucleotide-binding (G protein)-coupled receptors (LGR) previously known as LGR7 and LGR8 (Hsu et al., 2002), which have since been renamed as Relaxin Family Peptide (RXFP) RXFP1 and RXFP2, respectively. The human relaxin H2 activates both RXFP1 and RXFP2 resulting in an increase in intracellular cAMP concentrations (Dessauer and Nguyen, 2005; Halls et al., 2006; Nguyen and Dessauer, 2005; Nguyen and Dessauer, 2005; Nguyen et al., 2003) in THP-1, MCF-7 and HEK cells. Although the signaling by relaxin in fibroblastic cells has not yet been well characterized, in rat renal and cardiac fibroblasts nitric oxide instead of cAMP appears to be the major relaxin H2 signaling molecule (Samuel et al., 2004).

Both relaxin receptors and their transcripts have been identified in reproductive and non-reproductive tissues such as the brain, kidney, lung, anterior cruciate ligament of the knee joint and in synovial joint fibrocartilaginous cells (Faryniarz et al., 2006; Hsu et al., 2002; Wang et al., 2009). Although the precise contributions of RXFP1 and RXFP2 to modulation of MMPs upon activation by relaxin are not known, indirect evidence for the role of RXFP1 to *in vivo* remodeling of matrices is provided by the phenotypic characteristics of the female RXFP1 null mice that are similar to those described for relaxin-deficient mice (Kamat et al., 2004; Krajnc-Franken et al., 2004; Zhao et al., 1999). Although relaxin binds to both RXFP1 and 2, further indirect evidence that RXFP1 rather than RXFP2 is the likely candidate receptor for MMP regulation by relaxin are suggested by findings showing that the latter is a known cognate receptor for Insulin3 (INSL3) peptide (Bogatcheva et al., 2003; Del Borgo et al., 2006; Kumagai et al., 2002), and that the phenotypes of mice with INSL3 or RXFP2 mutations have little in common with those with relaxin-1 or RXFP1 deficiency (Ivell et al.,

2011; Kamat et al., 2004; Krajnc-Franken et al., 2004; Samuel et al., 2004; Samuel et al., 2005; Samuel et al., 2005). Finally, recent studies have demonstrated that relaxin-3 also modulates tissue remodeling in a manner similar to that by relaxin H2 through RXFP1 and that human relaxin-3 does not activate RXFP2 (Hossain et al., 2011; Samuel et al., 2007; Samuel et al., 2007). These findings taken together indirectly, but not conclusively, demonstrate that the tissue remodeling by relaxin likely occurs through RXFP1 rather than RXFP2. While these studies suggest that RXFP1 is a likely candidate receptor in the modulation of tissue remodeling, its role and that of RXFP2 in the induction of MMPs by relaxin has not been determined. Furthermore, although relaxin is known to modulate several signaling pathways on activating RXFP1 or RXFP2 (Halls et al., 2005; Halls et al., 2006; Halls et al., 2007; Halls et al., 2009), the cascade of signals that lead to relaxin's induction of MMPs by one or both of these receptors have not been determined.

In this investigation we sought to determine the precise contributions of RXFP1 and RXFP2 to the regulation of MMP-9, and -13, and to elucidate the downstream signaling pathways from the receptors in the induction of MMP-9 in fibrochondrocytes from a mouse synovial joint. We chose to investigate the mechanisms of relaxin's regulation of MMPs in synovial joint fibrochondrocytes since the induction of MMP-9 and -13 by relaxin as well as relaxin receptor expression has been well characterized in this cell system (Hashem et al., 2006; Kapila, 1997; Kapila, 2003; Kapila et al., 1995; Kapila et al., 2009; Kapila and Xie, 1998; Wang et al., 2007). We also studied the regulation of MMP-14 by relaxin since this proteinase, like MMP-13 is a collagenase, but is regulated substantially differently than the other collagenases (Chakraborti et al., 2003; Yan and Boyd, 2007) thus serving as suitable control. Our results show that the relaxin H2 induces MMP-9 and -13 in fibrochondrocytes through the RXFP1 receptor, and that relaxin's modulation of MMP-9 occurs via PI3K-AKT-PKCζ-ERK1/2 signaling pathway and involves Elk1 and c-fos transcription factors. These findings provide the first characterization of signaling cascade involved in the regulation of any MMP by relaxin and offer critical mechanistic information on the relaxin-mediated turnover of the ECM in fibrocartilaginous cells.

2. Material and Methods

2.1 Reagents and animals

All cell culture reagents and media were purchased from Invitrogen Corp. (Carlsbad, CA) and chemicals were from Sigma-Aldrich Corp. (St. Louis, MO) unless otherwise mentioned. Recombinant human relaxin-2 was a gift from BAS Medical (San Mateo, CA). C57BL/6J female mice were obtained from Charles River Laboratories (Wilmington, MA).

2.2 Fibrochondrocyte Isolation and Culture

Temporomandibular joint (TMJ) disc fibrochondrocytes were isolated from 12-week-old female C57BL/6J mice as described previously (Wang et al., 2009) and cultured in α-MEM supplemented with 10% fetal bovine serum (FBS). The doses of siRNA, cDNA signaling inhibitors and optimal timeframe for each experiment were determined by preliminary dose-response and time course studies. A minimum of three early passage (P2 to P4) fibrochondrocyte preparations were used for each experiment.

2.3 Overexpression of Relaxin Receptors

The fibrochondrocytes were seeded at 1.0×10^6 cells / 6 cm dish and transfected after 16 hours with 2μg of RXFP1 cDNA, or RXFP2 cDNA (Hsu et al., 2000; Hsu et al., 2002) (both kindly provided by Dr Teddy Hsu) or control pcDNA vector (Qiagen, Valencia, CA) using Effectene transfection reagent according to the manufacturer's instructions (Qiagen) in serum-free Opti-MEM media, with about 40 to 60% transfection efficiency. After 6 hours of

incubation, the Opti-MEM was replaced with α-MEM containing 20% FBS plus antibiotics (1% penicillin/streptomycin) and maintained for a period of 12 hours. The cells were then washed and maintained in serum-free medium (α-MEM with 0.2% lactalbumin hydrolysate; LAH) for 4 hours, before being incubated in fresh serum-free medium with or without 0.1ng/ml relaxin. We used 0.1ng/ml of relaxin because it induces maximum levels of MMPs in fibrochondrocytes (Kapila and Xie, 1998), and because this is within the range of physiologic concentrations of relaxin in serum of cycling women (Petersen et al., 1995; Stewart et al., 1990). After 48 hours of incubation cell conditioned media, cell lysates, or mRNA were collected and stored at −80°C until assayed.

2.4 Suppression of Relaxin Receptors

The fibrochondrocytes were seeded at 1.0×10^6 cells per 6 cm dish and transfected after 16 hours with 250 pm RXFP1 or RXFP2 or control siRNA (sc-40178, sc-40180 and sc-37007, respectively; Santa Cruz Biotechnology, Santa Cruz, CA) using Lipofectamine 2000 (Invitrogen Corp.) in serum-free Opti-MEM media according to the manufacturer's instructions. The siRNA combinations used are products of three target specific 20–25 nuleotide siRNAs. Lipofectamine was used to transfect siRNA since it provided adequate knockdown of the receptors of 50% or more, while being compatible with cell survival and health throughout the duration of the experiment. The remaining procedures were as described for the relaxin receptor overexpression experiments.

2.5 Analyses of Signaling Responses

Since the PI3K, PKC-ζ, and ERK signaling pathways that are modulated by relaxin converge on regulatory promoter elements, ETS/Elk-1, NF-κB, AP-1 that are common to several MMPs including MMP-1, -9 and -13, these candidate molecules were the focus of our studies. The fibrochondrocytes were seeded at 1.0×10^6 cells / 6 cm dish in α-MEM medium with 10% FBS. After 12 hours cells were washed and maintained in serum-free medium (α-MEM with 0.2% LAH) for 4 hours, following which fresh serum-free medium containing one of the following signaling inhibitors was added: 1mM PI3K inhibitor (LY294002)), 1mM general PKC inhibitor (Chelerythrine Chloride), 2mM PKC-ζ inhibitor (myristoylated PKC-ζ pseudo substrate) (All from EMD Biosciences Gibbstown, NJ), 1mM insulin inhibitor (Tyrphostin; Santa Cruz Biotechnology Inc.) or 1mM MEK/ERK1/2 inhibitor (U0126; Santa Cruz Biotechnology). After 30 minutes, the cells were incubated in the absence or presence of 0.1ng/ml relaxin. Cell lysates were collected after 20 minutes for assays on signaling molecules, while conditioned media were collected after 6 hours for MMP-9 assays. The time-points for these assays were selected on the basis of findings from preliminary studies. The conditioned media was concentrated using Amicon Ultra 10K centrifugal units (Millipore, Billerica, MA). Cell lysates were prepared in RIPA lysis buffer supplemented with proteinase inhibitors (Calbiochem, San Diego, CA) and 1mM Na3VO4, 1mM NaF. The lysates and conditioned media were assayed for total protein by BCA protein assay (Pierce Biotechnology, Rockford, IL) according to manufacturer's instructions. The levels of phosphorylated and total isoforms of candidate signaling molecules or MMP-9 were determined by Western blotting.

2.6 MMP-9 Promoter Regulation via Akt Actication

We next used -1017 bp MMP-9 promoter-luciferace construct containing all the important elements including three AP1, three NF-κB, one ETS and other sites required for MMP-9 regulation (Tacon et al., 2010) in conjunction with Akt dominant negative construct to confirm the contribution of relaxin-mediated Akt signaling to MMP-9 induction. The Akt dominant negative construct has mutated phosphorylation sites whereby the lysines in the ATP binding pocket have been substituted with alanine resulting in complete loss of kinase activity (Stambolic et al., 1998).

The fibrochondrocytes were seeded at 1.0×10^6 cells / 6 cm dish for 16 hours and transfected with Akt dominant negative construct (100 to 200 ng) (kindly provided by Dr. Inoki) or control vector, together with or without MMP-9 promoter-luciferase construct (0.25 μg) in PGL4 vector (kindly provided by Dr. Leigh) or PGL4 basic vector (Promega Corp., Madison, WI) using Effectene transfection reagent according to the manufacturer's instructions (Qiagen Corp.) in serum-free Opti-MEM media. pCMV vector containing β-galactosidase (Clontech, Mountain View, CA) was used as a control to standardize for transfection efficiency. After 6 hours of incubation, the Opti-MEM was replaced with α-MEM containing 20% FBS plus antibiotics (1% penicillin/ streptomycin) and maintained for a period of 12 hours. The cells were washed and maintained in serum-free medium (α-MEM with 0.2% LAH) for 4 hours before being incubated in fresh serum-free medium with or without 0.1ng/ml relaxin. After 6 hours of incubation cell lysates were collected and assayed using luciferase assay system (Promega Corp) as per the manufacturer's instructions. β-galactosidase assayed with the Tropix assay reagent (Applied Biosystems, Foster City, CA) was used for standardization of luciferase activity. Cell conditioned medium was collected and subjected to Western blot for MMP-9.

2.7 Silencing of PKC-ζ, ERK1/2, Elk-1 and NF-κB Signaling Molecules

The fibrochondrocytes were seeded at 1.0×10^6 cells per 6 cm dish and after 16 hours were transfected with 250 pm PKC-ζ, ERK1/2, Elk-1 or NF-κB or control siRNA (sc-36254, sc-29308/sc-35336, sc-35291 sc-29411 and sc-37007; Santa Cruz Biotechnology) using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen Corp.) in serum-free Opti-MEM media. The remaining procedures were as described for the relaxin receptor suppression experiments.

2.8 Western Blots

Western blots were used to evaluate changes in RXFP1, RXFP2 and MMP-14 in cell lysates, and MMP-9 and -13 in cell conditioned medium. The cell lysates or conditioned media, standardized for protein, were electrophoretically resolved on 10% SDS-PAGE gels and the proteins transferred to PVDF membranes. After blocking with 5% nonfat milk, the membranes were washed and incubated with one of the following primary antibodies: rabbit anti-human RXFP1 antibody (1:500 dilution; Phoenix Pharmaceuticals, Burlingame, CA), goat anti-human RXFP2 antibody (1:2000 dilution; Santa Cruz Biotechnology), rabbit anti-human MMP-9 (1:2000 dilution; Sigma Chemicals), rabbit anti-human MMP-13 (1:500 dilution; Abcam Inc. Cambridge, MA) or rabbit anti-human MMP-14 (1:500 dilution; Abcam Inc.). The membranes were washed followed by addition of appropriate horseradish peroxidase- (HRP-) linked secondary antibody (1:3000 dilution; Santa Cruz Biotechnology) for two hours. The membranes were washed and the respective protein bands were visualized using enhanced chemiluminescence (SuperSignal® West Pico, Thermo Scientific, Rockford, IL). To confirm equal loading of the cell lysate, membranes were treated with Restore™ Western Blot Stripping Buffer (Thermo Scientific) for 15 minutes, washed, blocked and re-probed with rabbit anti-mouse antibody specific for actin (1:2000 dilution; Santa Cruz Biotechnology). The blot was then developed as described above.

Western blots for detection of signaling molecules and MMP-9 in the signaling experiments were performed as described above except that the chemiluminescence reagents were supplemented with 5% enhancer solution (SuperSignal® West Femto, Thermo Scientific) for visualization of MMP-9 and phosphorylated isoforms of p-PI3K, p-Elk-1, p-Akt, p65-NF-κB p-ERK1/2 and p-cfos proteins. The membranes were incubated overnight with one of the following rabbit anti-mouse antibodies: Akt (1:1000 dilution; Abcam Inc.), phospho-Akt, phospho-PI3K or PI3K, ERK 1/2, phospho-ERK 1/2, phospho-PKC-ζ, PKC- ζ, phospho-Elk-1, Elk-1 (1:1000 dilution; all from Cell Signaling Technology, Danvers, MA),

phospho-P65-NF-κB, and P65-NF-κB (1:200 dilution; Santa Cruz Biotechnology Inc.). The blots were then developed as described above.

2.9 Quantitative reverse transcription-PCR (qRT-PCR)

Trizol reagent (Invitrogen Corp.) was used according to the manufacturer's directions to extract total RNA from the cells. Any DNA contaminations were removed by using DNA *free*™ kit (Invitrogen Corp.). Total RNA (1μg) from each sample was reverse-transcribed using Superscript™ First-Strand Synthesis System for RT-PCR (Invitrogen Corp.) as per manufacturer's instructions. qRT-PCR utilizing TaqMan® Universal PCR Master Mix (Applied Biosystems) was used to confirm the findings of the RT-PCR assays and to quantify relaxin receptors and MMP -9 and -13 mRNA. All probes specific for mouse or human RXFP1 and RXFP2, mouse MMP-9 and MMP-13, and the internal control β-Actin were obtained commercially (TaqMan Assays on Demand, Applied Biosystems). Amplification of 1:10 diluted cDNA was conducted under the following conditions: 50 °C for 2 min; 95 °C for 10 min, followed by 50 cycles at 94 °C, 15 s, and 60 °C for 1 min. The relative amount of the products was determined by using the ABI Prism 7500 SDS 1.2 Software (Applied Biosystems).

2.10 Statistical Analysis

All experiments and assays were performed in triplicate and the outcomes of quantitative data presented as means and corresponding standard errors. Western blots were quantitated by videodenistometry and the data standardized by total protein, actin or unphosporylated protein presented as mean (+/− SE) fold change. The effects of suppression or induction of receptors, and the inhibition of signaling molecules or transcription factors on activation of signaling pathways or MMP induction were analyzed statistically by ANOVA. Intergroup differences were analyzed by a post-hoc t-test with a level of significance set at $p<0.05$.

3. Results

3.1 Overexpression of RXFP1 but not RXFP2 enhances relaxin's induction of MMP-9 and -13; MMP-14 is not regulated by relaxin

We first validated the ability of cDNA transfections to enhance the expression of RXFP1 and RXFP2, assessed the effects of relaxin on the expression of these receptors, and determined the contribution of either receptor to relaxin's modulation of MMP-9, -13 and -14. Transfection of the cells with RXFP1 and RXFP2 cDNAs resulted in a successful statistically significant overexpression ($p<0.05$) of the receptors at the protein (Figs. 1A and 2A, lane 3 vs. lanes 1 and 2; lane 6 vs. lanes 4 and 5; Figs. 1B and 2B) and mRNA (Figs. 1C and 2C) levels relative to untransfected control cells and empty vector transfected cells. Quantitation of the receptor levels by qRT-PCR showed that cDNA transfection resulted in a four-fold ($p<0.05$) higher expression of RXFP1 mRNA (Fig. 1C) and an eight-fold ($p<0.05$) higher RXFP2 mRNA expression (Fig. 2C) relative to control and empty vector samples. The lower fold-change in RXFP1 over RXFP2 following the respective cDNA transfections is likely due to higher baseline levels of RXFP1 than RXFP2 in these cells (Wang et al., 2009). Treatment of the cells with relaxin did not have any substantial effect on mRNA and protein expression levels of RXFP1 or RXFP2 (Figs. 1A to C, 2A to C), demonstrating that relaxin does not modulate the expression of these receptors in untransfected, empty vector transfected and cDNA transfected fibrochondrocytes.

We next assessed the ability of relaxin to induce MMP-9, -13 and -14 in control and receptor cDNA transfected cells. We found that relaxin induces MMP-9 and -13, but not MMP-14 in fibrochondrocytes (Figs. 1D to G and 2D to G), which is likely attributable to the presence of one or both receptors that are known to be endogenously expressed by these cells (Wang

et al., 2009). Relaxin treatment of the RXFP1 cDNA-transfected cells resulted in a statistically significant ($p<0.05$; Figs. 1E and F) potentiation of relaxin's induction of MMP-9 and -13 relative to their induction by relaxin in untransfected control or empty vector transfected control (Fig. 1D; lane 6 vs lanes 4 and 5) cells. In contrast to the findings in RXFP1 transfected cells, RXFP2 cDNA transfected cells showed no potentiation of MMP-9 and -13 by relaxin relative to control untransfected and empty vector transfected cells (Figs. 2D; lane 6 vs lanes 4 and 5; Figs. 2E and F). The findings that enhanced expression of RXFP1 but not RXFP2 potentiates the induction of MMP-9 and -13 by relaxin implicate RXFP1 in the induction of MMP-9 and -13 by relaxin in fibrochondrocytes.

3.2 Silencing of RXFP1 but not of RXFP2 decreases relaxin's induction of MMP-9 and -13

TMJ fibrochondrocytes express substantially higher baseline levels of RXFP1 than of RXFP2 (Wang et al., 2009) such that competitive binding of the ligand to the more highly expressed receptor may mask the contribution of RXFP2 to the modulation of MMPs by relaxin even when RXFP2 is overexpressed. Thus the findings of experiments in which the receptors are overexpressed may not provide conclusive evidence on the precise contribution of RXFP2 to relaxin's regulation of these MMPs. We therefore performed studies in which we examined the effects of suppression of each of these receptors on relaxin's modulation of MMPs to validate the role of RXFP1 and decipher the contribution of RXFP2 to modulation of MMPs by relaxin.

Transfection of the cells with siRNA to RXFP1 or RXFP2 resulted in successful and statistically significant suppression ($p<0.05$) of protein of the respective receptors (Figs. 3A and 4A; lane 3 vs. lanes 1 and 2; lane 6 vs. lanes 4 and 5; Figs. 3B and 4B). Quantitative RT-PCR analysis demonstrated a statistically significant 60% inhibition ($p<0.05$) of RXFP1 (Fig. 3C) and 70% inhibition ($p<0.05$) of RXFP2 (Fig. 4C) transcripts by their respective siRNAs. As with the previous experiments, treatment of the cells with relaxin did not modulate the levels of RXFP1 or RXFP2 both at transcriptional and translational levels (Figs. 3A to C, 4A to C).

As with the previous experiment, treatment of untransfected and empty vector transfected cells with relaxin resulted in significant increases ($p<0.05$) in translation of MMP-9 and -13, but not that of MMP-14 (Figs. 3C and 4C; lanes 1 and 2 vs. lanes 4 and 5, respectively; Figs. 3E to G and 4E to G). Suppression of RXFP1 resulted in a significant decrease ($p<0.05$) in the levels of constitutively expressed MMP-9 and -13 (Figs. 3C; lanes 1 and 2 vs. lane 3; Figs. 3E and F). Suppression of RXFP1 also significantly mitigated ($p<0.05$) relaxin's induction of MMP-9 and -13 relative to that in untransfected control vector transfected cells (Figs. 3C; lane 6 vs. lanes 4 and 5; Figs. 3E and F). Under the same conditions, suppression of RXFP2 did not result in any changes in relaxin's induction of MMP-9 and 13 (Figs. 4D, lanes 4 and 5 vs. lane 6; Figs. 4E and F). Taken together these findings demonstrate conclusively that RXFP1 is essential to the induction of MMP-9 and -13 by relaxin in fibrochondrocytes, while RXFP2 does not contribute to the modulation of these MMPs by relaxin.

3.3 Changes in MMP-9 and -13 transcript levels in relaxin receptor-overexpressed and suppressed cells confirms RXFP1 as the primary relaxin receptor in their modulation

The final evidence for the prominent role of RXFP1 receptor in modulating the expression of MMP-9 and -13 transcripts was obtained by MMP qRT-PCR assays in RXFP1 and RXFP2 overexpressed or knockdown cells. Because of the presence of endogenous relaxin receptors on the fibrochondrocytes (Wang et al., 2009), relaxin significantly upregulated ($p<0.05$) the expression of MMP-9 and -13 mRNAs by 3- and 4-fold respectively when compared to untreated control empty vector transfected cells (Figs. 5A and B).

Overexpression of RXFP1 resulted in a 1.6 and 2.1-fold increase in constitutively expressed MMP-9 and -13, respectively, while overexpression of RXFP2 resulted in no change in mRNA transcripts of these MMPs. Overexpression of RXFP1 resulted in a significant potentiation ($p<0.05$) of the induction of MMP-9 and -13, respectively, by relaxin relative to cells transfected with empty control vector. Relaxin treatment of cells overexpressing RXFP2 did not modulate any changes in the relaxin-stimulated levels of MMP-9 and -13 mRNAs.

Suppression of RXFP1 resulted in a significant 45% and 60% decrease ($p<0.05$) in the constitutive levels of MMP-9 and -13 mRNAs, respectively (Figs. 5C and D). In contrast, suppression of RXFP2 had no effect on the mRNA transcript levels for either proteinase. As with previous experiments, exposure of the cells to relaxin resulted in a 3- and 4-fold increase in MMP-9 and -13 mRNAs, respectively. This induction of MMP-9 and -13 by relaxin was downregulated by 51% and 56% ($p<0.05$) respectively in RXFP1 silenced cells relative to relaxin-treated empty vector cells. In contrast, suppression of RXFP2 resulted in no significant change in relaxin-stimulated expression of MMP-9 and -13. These results confirm the involvement of RXFP1 but not RXFP2 in the regulation of MMP-9 and -13 by relaxin in fibrochondrocytes.

3.4 RXFP1-mediated induction of MMP-9 occurs via PI3K-PKCζ-ERK1/2 pathway

Because we demonstrated that RXFP1 and not RXFP2 is the primary receptor involved in the regulation of MMP-9 and -13 by relaxin, in the next series of experiments we dissected the downstream signaling cascade from RXFP1 involved in the induction of MMP-9 by relaxin. We chose to determine this signaling cascade for MMP-9 rather than MMP-13 since the former proteinase is more easily detected and assayed for than MMP-13 in fibrochondrocyte cell system. For these studies, we focused on the currently known signaling pathways that are modulated by RXFP1 and specifically those that are likely to converge on the known transcription promoter/start sites of the MMP-9 promoter (Gum et al., 1997; Hussain et al., 2002). We thus used inhibitors to PI3K (LY294002), PKC (Chelerythrine chloride) and PKC-ζ (myristoylated pseudo substrate) to determine the contribution of these molecules to relaxin's induction of MMP-9. Because ERK1/2 can be an immediate target of PI3K signaling molecule and because MMP-9 can be unregulated by ERK1/2 pathway, we also assayed the effects of MEK/ERK1/2 inhibitor U0126 on MMP-9 regulation by relaxin. Tyrphostins, an inhibitor for the insulin signaling pathway (Parrizas et al., 1997), was used as a negative control.

The immunoblot of the conditioned media obtained from fibrochondrocytes pretreated with PI3K, PKC PKC-ζ and ERK inhibitors showed that these signaling molecules are involved in the induction of MMP-9 by relaxin (Fig. 6A). Specifically, relaxin treated samples showed an increase in MMP-9 expression relative to untreated samples (Fig. 6A, lanes 2 and 4 vs. lanes 1 and 3), and this induction was decreased to or below basal levels when cells were exposed to the inhibitors for PI3K, PKC and PKC-ζ, but not with the inhibitor for insulin signaling Tyrphostins. MEK/ERK1/2 inhibitor U0126 also blocked relaxin's induction of MMP-9. The expression of MMP-9 in lanes 9, 10, 12 and 14 was very low, demonstrating a potent effect of PKC-ζ and MEK/ERK1/2 inhibitors in mediating not only relaxin's induction of MMP-9, but also the constitutive expression of MMP-9 in these cells. The inhibitors also significantly decreased ($p<0.05$) the levels of phosphorylated forms of their respective signaling molecules and that of downstream signaling molecules that paralleled their inhibition of MMP-9 induction by relaxin (Figs. 6A and B). Since both ERK and Akt are downstream of PI3K activation, and because Akt signaling has been shown to be involved in MMP-9 upregulation (Byun et al., 2006; Miyamoto et al., 2005; Yoo et al., 2011), we also determined if relaxin activates Akt. We found that relaxin enhanced the phosphorylation of Akt, and that this phosphorylation was inhibited in the presence of PI3K

inhibitor concurrent with the lack of induction of MMP-9 by the hormone (Figs. 6A and B). These findings demonstrate that relaxin's induction of MMP-9 occurs by PI3K-PKCζ-ERK1/2 pathway and also involves Akt phosphorylation.

3.5 Relaxin's induction of MMP-9 is Akt dependent

Since the previous findings suggested that Akt is involved in MMP-9 induction by relaxin and because the RXFP1 activation of ERK1/2 can be either Akt dependent or independent (Vivanco and Sawyers, 2002), we used Akt dominant negative constructs to confirm whether Akt is indeed involved in the relaxin's induction of MMP-9. Relaxin treatment of cells transfected with control vector(s), or with Akt dominant negative constructs and/or MMP-9 promoter-luciferase constructs showed that the baseline and relaxin-stimulated activation of MMP-9 promoter was significantly reduced (*$p<0.05$) in samples cotransfected with Akt dominant negative constructs (Fig. 7A). Concomittant with this suppression of MMP-9 promoter, the expression of endogenous MMP-9 was also dose-dependently inhibited in cells transfected with the Akt dominant negative construct (Fig. 7B) thereby confirming that Akt is an important signaling molecule in relaxin's induction of MMP-9 likely via ERK1/2 phosphorylation.

3.6 RXFP1 activated PI3K-PKCζ-ERK pathway increases the phosphorylated forms of Elk-1 and NF-κB transcriptional factors concurrent with modulation of MMP-9

Since Elk-1, NF-κB and c-fos/c-jun transcription factors are downstream targets of activated ERK and PKC-ζ that we demonstrated to be upregulated by relaxin and that contribute to its induction of MMP-9 (Figs. 6A and B), and because these molecules have previously been shown to transactivate MMP-9 expression (Duxbury and Whang, 2007; Ho et al., 2007; Onodera et al., 2002; Wang et al., 2010; Wang et al., 2010), we next determined the phosphorylation status of P65-NF-κB, Elk-1 and c-fos upon stimulation of the fibrochondrocytes by relaxin. We found that relaxin treatment of the fibrochondrocytes enhanced phospho forms of Elk-1, c-fos and P65-NF-κB, indicating a possible role of these transcription factors in the induction of MMP-9 (Fig. 8B). Inhibition of the activation of PI3K and ERK, and to a lesser extent of PKC, resulted in decrease in the levels of relaxin-mediated phosphorylation of Elk-1 and c-fos suggesting that Elk-1 and c-fos are the downstream targets of PI3K and ERK. The activation of NF-κB by relaxin was dependent on PKC and PKC-ζ activation, but independent of ERK phosphorylation. Additionally, inhibition of the PKC and PKC-ζ also diminished ERK phosphorylation demonstrating a second pathway for ERK activation (Fig. 6B). Overall these results reveal that treatment of relaxin culminates in activating the Elk-1, c-fos and NF-κB transcription factors, which in turn upregulate MMP-9 gene expression.

3.7 Silencing of ERK1/2, PKC-ζ, Elk-1, c-fos and to a lesser extent of NF-κB abrogates the induction of MMP-9 by relaxin

We next confirmed the results of chemical inhibitor studies on relaxin-mediated phosphorylation of Elk-1 and NF-κB, and dissected the role that the activation of these transcriptional factors and their respective upstream signaling molecules play in relaxin's induction of MMP-9. For these experiments, we used cells in which ERK1/2, PKC-ζ, Elk-1 or NF-κB was suppressed using siRNA. Transfection of the cells with siRNA markedly reduced the total ERK1/2, PKC- ζ, Elk-1, and NF-κB proteins (Figs. 9A to D, lanes 1 and 2 vs. 3 and lanes 4 and 5 vs. 6). Relaxin treatment resulted in increased levels of phosphorylated ERK1/2, PKC- ζ, Elk-1, and NF-κB (Fig. 9, lanes 1 and 2 vs. 4 and 5). Relaxin's phosphorylation of these transcription factors was significantly (*$p<0.05$) suppressed in cells transfected with the respective siRNAs (Fig. 9, lane 6 vs. lanes 4 and 5). Furthermore, transfection of cells with siRNA for ERK1/2, PKC-ζ or Elk-1 and to a lesser extent for NF-κB abrogated the induction of MMP-9 by relaxin (Fig. 9, lane 6 vs. lanes 4

and 5). Silencing of NF-κB only minimally reduced relaxin's induction of MMP-9 expression in fibrochondrocytes (Fig. 9D, lane 6 vs. lanes 4 and 5). These findings demonstrate that ERK1/2, PKC-ζ and Elk-1 are likely to be the primary transcriptional factors while NF-κB plays a smaller role in relaxin's induction of MMP-9.

4. Discussion

These studies show for the first time that relaxin induces MMP-9 and -13 through activation of RXFP1 but not via RXFP2. We also show that MMP-14 is not regulated by relaxin. Our findings also provide the first characterization of signaling cascade involved in the regulation of any MMP by relaxin, and present evidence for the involvement of the P13K, Akt, ERK and PKC-ζ signaling pathways, as well as Elk-1 and c-fos transcription factors in relaxin's induction of MMP-9. We also noted that the overexpression or suppression of RXFP1 resulted in the transcriptional upregulation or transcriptional and translational suppression, respectively of constitutively expressed MMP-9 and -13. The reasons for the differences of the effects of overexpression of RXFP1 on MMP mRNA vs protein levels may be attributed to the timing and compartments in which these two products were assayed. Thus MMP mRNA assayed from the cell extract at 48 hours of culture represents stable mRNA present at that particular time point, whereas the protein assayed in cell conditioned medium represents cumulative levels of protein through the experimental period. Also, while the modulation of constitutive levels of these MMPs by overexpression or suppression of RXFP1 could have resulted from autocrine/paracrine activation by locally produced relaxin, we found that these cells do not express relaxin (data not shown). This suggests other unknown mechanisms may be involved in this response. One such possibility is recently reported constitutively active signalosome formed by an RXFP1 complex with AKAP79, AC2, β-arrestin-2 and PDE4D3 that acts differently from PI3K and PKC-ζ signaling pathways (Halls and Cooper, 2010). Whether this or an alternative mechanism is responsible for the constitutive regulation of MMP-9 and -13 by the overexpression or suppression of RXFP1 remains to be determined.

In vivo evidence in support of our findings on the relationship between relaxin-RXFP1-MMP axis and ECM remodeling is provided by studies showing that RXFP1 null mice have several abnormal characteristics in matrices that are similar to those described in relaxin-deficient mice. These include an increased local density of collagen in the nipple and an unrelaxed pubic symphysis that retains densely packed collagen fibers during parturition (Kamat et al., 2004; Krajnc-Franken et al., 2004; Zhao et al., 1999; Zhao et al., 2000). Furthermore, RXFP1 deficient mice have decreased levels of MMP-9 in lungs (Samuel et al., 2009) that, together with our findings, validate the contribution of RXFP1 in relaxin's regulation of specific MMPs. Finally, support for the physiologic relevance of our studies is provided by previous investigations showing the contribution of the relaxin-MMP axis to loss of collagen and glycosaminoglycans in joint fibrocartilage both in tissue explants and *in vivo* (Hashem et al., 2006; Naqvi et al., 2005). Thus, our current *in vitro* findings linking the activation of RXFP1 by relaxin and the subsequent downstream signals to the induction MMP provide a mechanistic basis on how relaxin regulates ECM phenotype *in vivo* and *ex vivo* (Hashem et al., 2006; Naqvi et al., 2005; Samuel et al., 1996; Samuel et al., 1998; Unemori et al., 1996).

While relaxin's activation of RXFP1 is known to modulate multiple signaling pathways that vary between target tissues (Bathgate et al., 2005; Halls et al., 2005; Halls et al., 2007; Summers et al., 2009), their subsequent downstream targets remain to be characterized. Conversely, although we have previously demonstrated that downstream events in the induction of the human MMP-1 by relaxin involves and requires the AP-1 and PEA-3 promoter sites in fibrocartilaginous cells (Kapila et al., 2009), the upstream and intermediate

signaling events involved in regulating MMPs by relaxin have not been elucidated to date. Studies so far have shown that the RXFP1 acts as a Gs-linked G-protein coupled receptor (GPCR) and signals through adenylate cyclase to increase cAMP in THP-1, MCF-7 and HEK cells (Bathgate et al., 2005; Bathgate et al., 2003; Summers et al., 2009). It has also been shown that RXFP1 can recruit $G\alpha_{i3}$ and release $G\beta\gamma$ thereby increasing cAMP in a delayed manner (Halls et al., 2006; Halls et al., 2009) through PI3K-PKC-ζ pathways (Dessauer and Nguyen, 2005; Nguyen and Dessauer, 2005; Nguyen and Dessauer, 2005; Nguyen et al., 2003). These findings together with ours on the involvement of PI3K-PKC-ζ signaling pathways in relaxin's induction of MMP-9 validate the contribution of RXFP1, a $G\alpha_s$ linked GPCR, to this cascade.

Our studies also show that the downstream events following relaxin's activation of PI3K include phosphorylation of Akt and ERK1/2 and the involvement of these molecules in the induction of MMP-9 by relaxin in the fibrocartilaginous cell system. PI3K and Akt can have multiple downstream signaling pathways. One of the important signaling pathways for PI3K involves the phosphorylation of ERK1/2, which in turn was found to be dependent on Akt activation. Because relaxin's induction of MMP-9 is decreased to basal levels by the inhibition of ERK both by ERK and by PI3K inhibitors demonstrates that the ERK signaling pathway is the one involved in PI3K modulation of MMP-9 in fibrochondrocytes. Furthermore, the inhibition of Akt phosphorylation by PI3K inhibitor concomitant with the abrogation of MMP-9 induction by relaxin as well as the downregulation of MMP-9 with Akt dominant negative construct suggests that this effect is mediated via Akt. Further support for the involvement of the ERK pathway in the regulation of MMP-9 by relaxin are provided by the finding that the activation of Elk-1 and c-fos, that are direct downstream targets of ERK1/2 signaling pathway, increase with relaxin treatment. Upon phosphorylation, Elk-1 and c-fos are known to translocate from the cytoplasm to the nucleus and are known to bind to ETS and AP-1 transcriptional motifs of the MMP-9 promoter, respectively and increase the transactivation of this proteinase (Kapila et al., 2009; Onodera et al., 2002; Wang et al., 2010; Wang et al., 2010). The findings that relaxin's induction of ERK1/2 phosphorylation during the differentiation of cardiac fibroblasts is independent of PI3K activation (Mookerjee et al., 2009) together with ours that ERK phosphorylation by relaxin is dependent on PI3K and Akt suggest that H2 relaxin can activate multiple signaling pathways in different cell systems.

In addition to activating ERK1/2, PI3K and Akt, relaxin treatment also enhances phosphorylation of PKC and PKC-ζ, whose immediate downstream signaling target is NF-κB (Diaz-Meco et al., 1994). PKC-ζ can phosphorylate the heterodimer of IκB and NF-κB thereby disrupting their association. P65-NF-κB translocates into the nucleus and can bind to the NF-κB regulatory element in the MMP-9 promoter. This suggests that in addition to Elk-1 activation of ETS promoter site, NF-κB may function as an alternative transcriptional factor for relaxin's induction of MMP-9. However, while the silencing of ERK1/2, PKC-ζ and Elk-1 eliminated relaxin's induction of MMP-9 thereby substantiating their significant role in relaxin's induction of MMP-9, the effect of silencing NF-κB on relaxin's induction of MMP-9 was less marked.

Our systematic analysis using specific chemical inhibitors and siRNAs has identified the requirement of Elk-1/ETS, c-fos and to a lesser extent NF-κB multiple regulatory elements for relaxin-mediated induction of MMP-9 that was earlier completely unknown (Fig. 10). Although previous studies have shown that the regulation of MMP-9 by hormones and growth factors involve ETS/PEA-3, NF-κB, GT box and AP-1 binding motifs of the MMP-9 promoter causing subsequent increase in MMP-9 transcriptional activity (Gum et al., 1997; Ray et al., 2005; Sato et al., 1993), the extent of involvement of these transcriptional factors in transactivating the MMP-9 expression is dependent on the cell type and the stimulus used.

The data from this study confirms that relaxin stimulation results in convergence of PI3K and PKC-ζ signaling pathways that activate the transcription factors c-fos, Elk-1, and NF-κB that likely bind to their respective motifs in the MMP-9 promoter to initiate the transcriptional activity of this gene. Since the inhibitors of PKC-ζ and ERK as well as silencing of these two factors were the most effective in eliminating relaxin's induction of MMP-9, it is possible that signals from these two pathways converge on common MMP-9 transcriptional motifs. This explains why blockage of a single pathway may abolish MMP-9 expression.

In conclusion our study shows that induction of MMP-9 by relaxin occurs via RXFP1 and that it involves more than one intracellular signaling network. These data provide insights and options for future clinical interventions for modulating the induction MMP-9 by relaxin in synovial joint fibrochondrocytes.

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Highlights

- Relaxin induces MMP-9 and -13 via RXFP1 and not RXFP2 in fibrochondocytes.
- Relaxin's induction of MMP-9 by RXFP1 activation is mediated via the PI3K, Akt, ERK and PKC-ζ signaling pathways.
- The transcription factors Elk-1 and c-fos are involved in up regulation of MMP-9 by relaxin.
- The findings provide insights into the mechanisms by which relaxin mediates the induction of MMP-9 and -13 that likely contribute to extracellular matrix turnover.

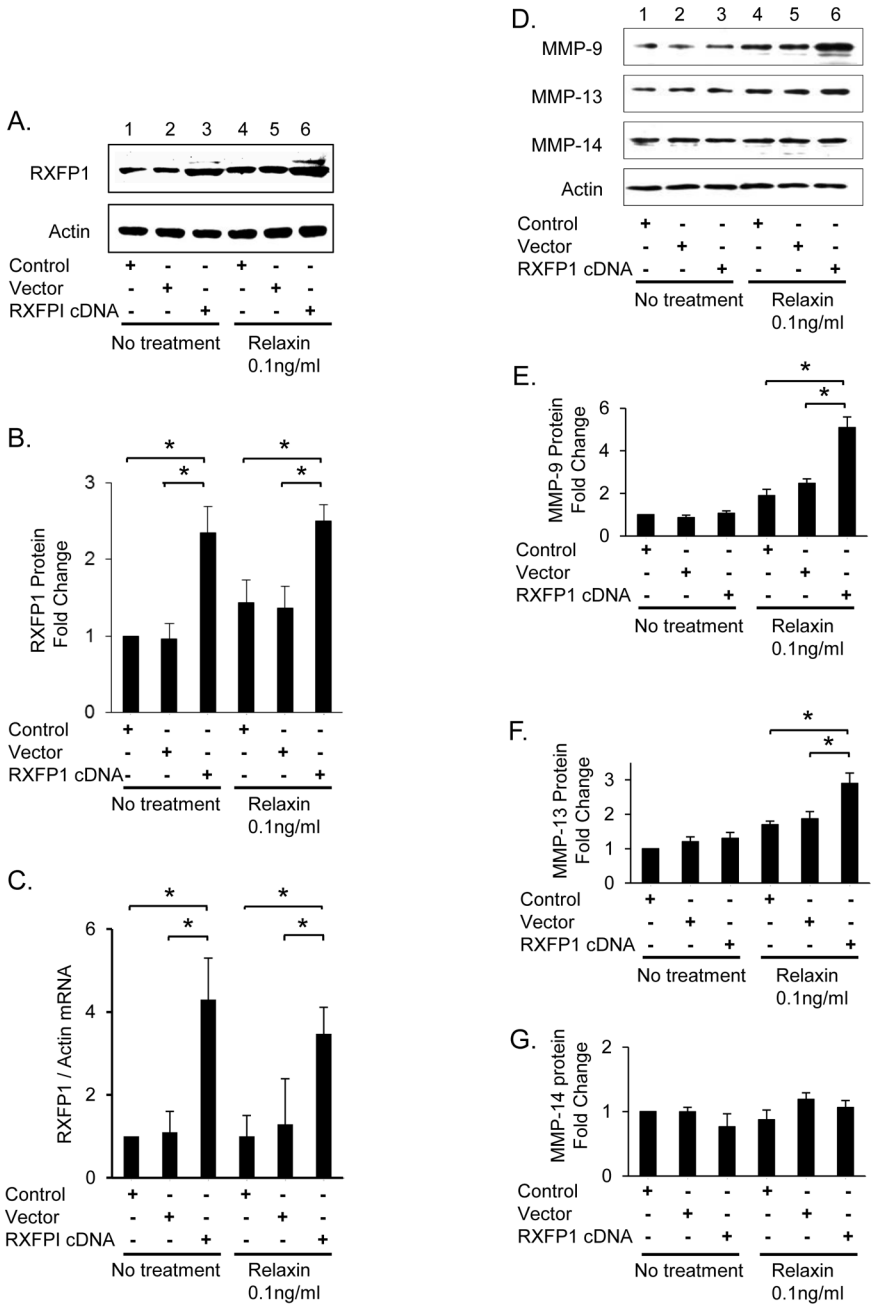

Fig. 1. Overexpression of RXFP1 potentiates relaxin's induction of MMP-9 and -13 in fibrochondrocytes

Untransfected cells (Control) or cells transiently transfected with pcDNA vector, or vector containing RXFP1 cDNA were cultured in the absence or presence of relaxin (0.1ng/ml). RNA, cell-matrix extract and cell-conditioned medium were collected after 48 hours and assayed. cDNA transfection successfully enhanced RXFP1 protein expression assayed by Western blots (A) and quantitated by videodensitometry (B), as well as mRNA assayed by qRT-PCR (C) in both untreated and relaxin-treated cells. Western blots for MMPs quantitated by videodensitometry revealed that relaxin induced MMP-9 (D and E) and -13 (D and F), but not MMP-14 (D and G). Cells overexpressing RXFP1 showed enhanced

induction of MMP-9 (D and E) and -13 (D and F) by relaxin. Quantitative data is shown as mean (±SE) fold-change in RXFP1 or MMP levels relative to control untransfected and untreated cells. Actin was used as a loading control for Western blots. (*$p < 0.05$).

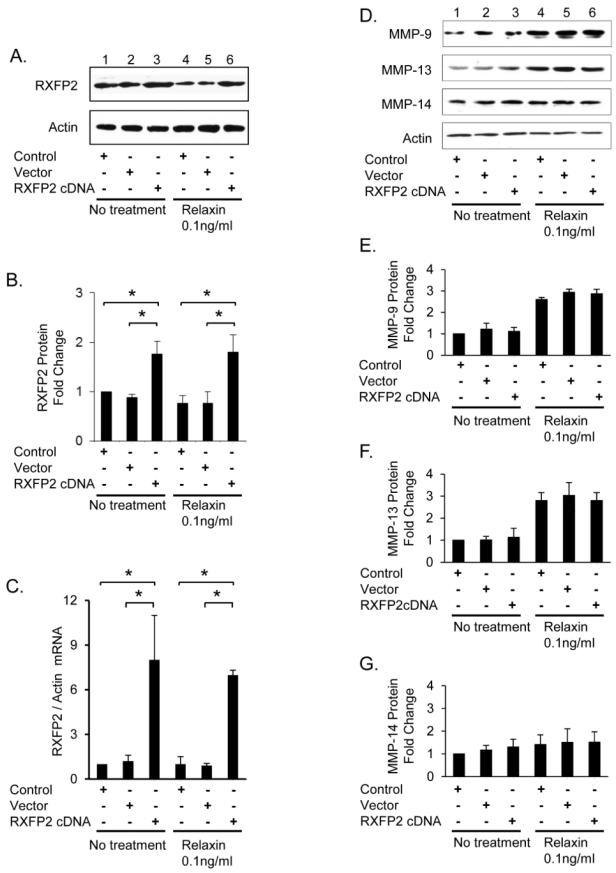

Fig. 2. Overexpression of RXFP2 does not modulate the expression of MMP-9 and -13 in fibrochondrocytes

Untransfected cells (Control) or cells transiently transfected with pcDNA vector, RXFP2 cDNA constructs were cultured in the absence or presence of relaxin (0.1ng/ml). RNA, cell-matrix extract and cell-conditioned medium were collected after 48 hours and assayed. cDNA transfection successfully enhanced RXFP2 protein expression assayed by Western blots (A) and quantitated by videodensitometry (B), as well as mRNA assayed by qRT-PCR (C) in both untreated and relaxin-treated cells. Western blots for MMPs quantitated by videodensitometry revealed that relaxin induced MMP-9 (D and E) and -13 (D and F), but not MMP-14 (D and G). Cells overexpressing RXFP2 showed no further modulation of MMP-9 (D and E) and -13 (D and F) by relaxin. Quantitative data is shown as mean (± SE) fold-change in RXFP2 or MMP levels relative to control untransfected and untreated cells. Actin was used as a loading control for Western blots. (*$p < 0.05$).

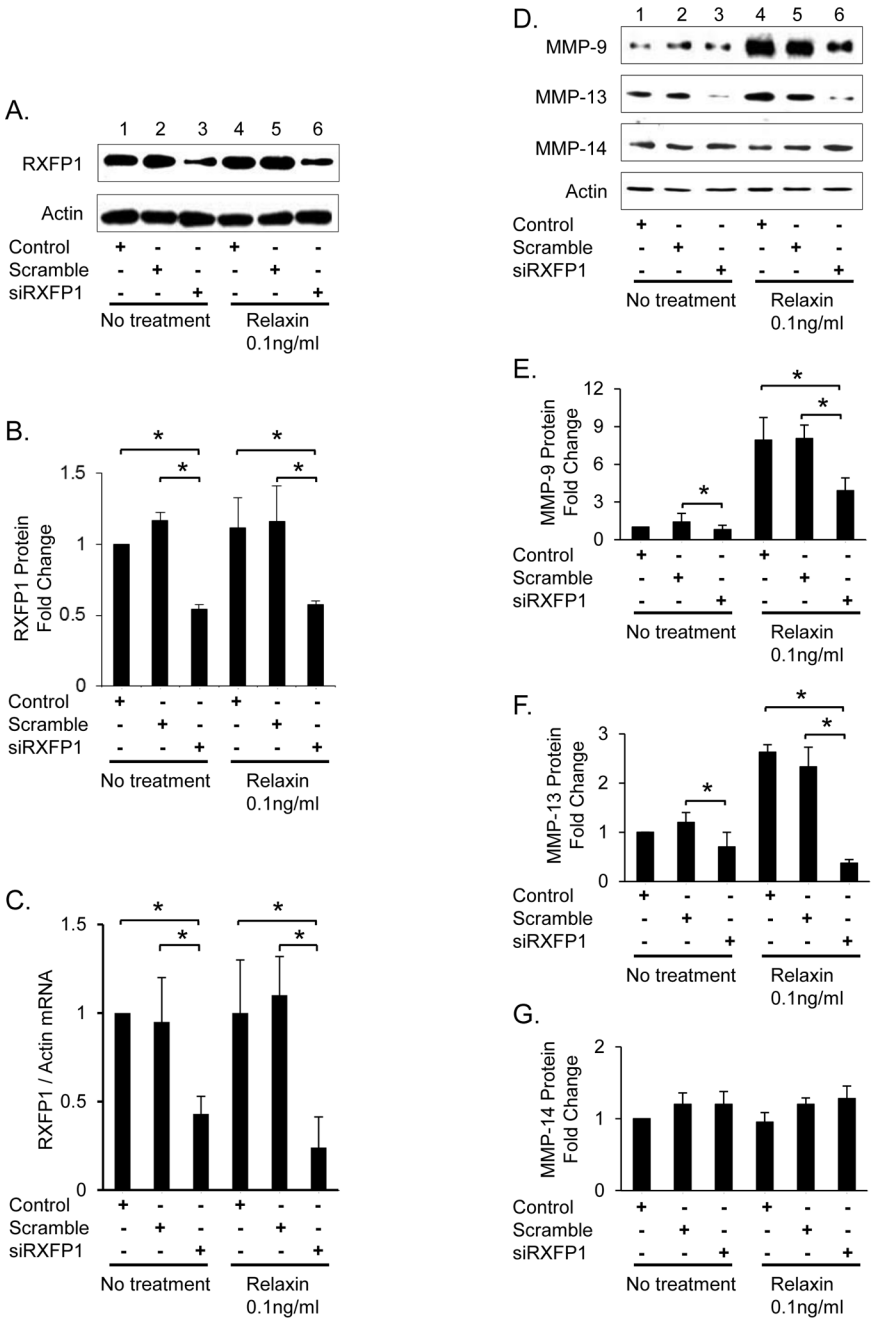

Fig. 3. Inhibition of RXFP1 expression negates the ability of relaxin to induce MMP-9 and -13 in fibrochondrocytes

Untransfected cells (Control) or cells transiently transfected with scrambled siRNA, or siRNA to RXFP1 were cultured in the absence or presence of relaxin (0.1ng/ml). RNA, cell-matrix extract and cell-conditioned medium were collected after 48 hours and assayed. siRNA transfections inhibited RXFP1 protein expression assayed by Western blots (A) and quantitated by videodensitometry (B), as well as mRNA assayed by qRT-PCR (C) in both untreated and relaxin-treated cells. Western blots for MMPs quantitated by videodensitometry revealed that relaxin induced MMP-9 (D and E) and -13 (D and F), but not MMP-14 (D and G). Suppression of RXFP1 decreased both the constitutive expression

levels and relaxin's induction of MMP-9 (D and E) and -13 (D and F). Quantitative data is shown as mean (±SE) fold-change in RXFP1 or MMP levels relative to control untransfected and untreated cells. Actin was used as a loading control for Western blots. (*$p < 0.05$).

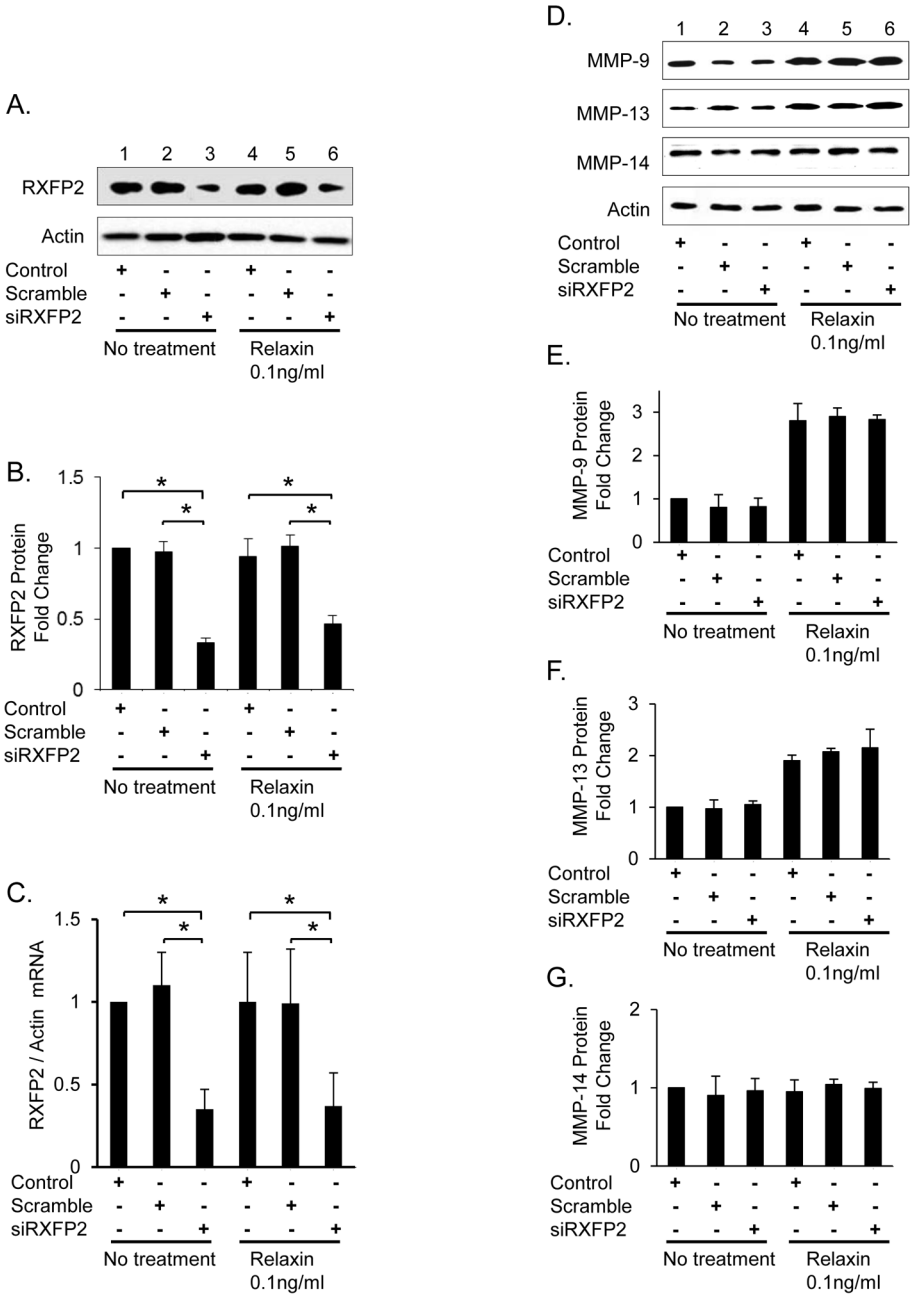

Fig. 4. Suppression of RXFP2 does not contribute to relaxin's modulation of MMP-9 and -13

Untransfected cells (Control) or cells transiently transfected with scrambled siRNA, or siRNA to RXFP2 were cultured in the absence or presence of relaxin (0.1ng/ml). RNA, cell-matrix extract and cell-conditioned medium were collected after 48 hours and assayed. siRNA transfections inhibited RXFP2 protein expression assayed by Western blots (A) and quantitated by videodensitometry (B), as well as mRNA assayed by qRT-PCR (C) in both untreated and relaxin-treated cells. Western blots for MMPs quantitated by videodensitometry revealed that relaxin induced MMP-9 (D and E) and -13 (D and F), but not MMP-14 (D and G). Suppression of RXFP2 did not modulate relaxin's induction of MMP-9 (D and E) and -13 (D and F). Quantitative data is shown as mean (±SE) fold-change

in RXFP1 or MMP levels relative to control untransfected and untreated cells. Actin was used as a loading control for Western blots. (*$p < 0.05$).

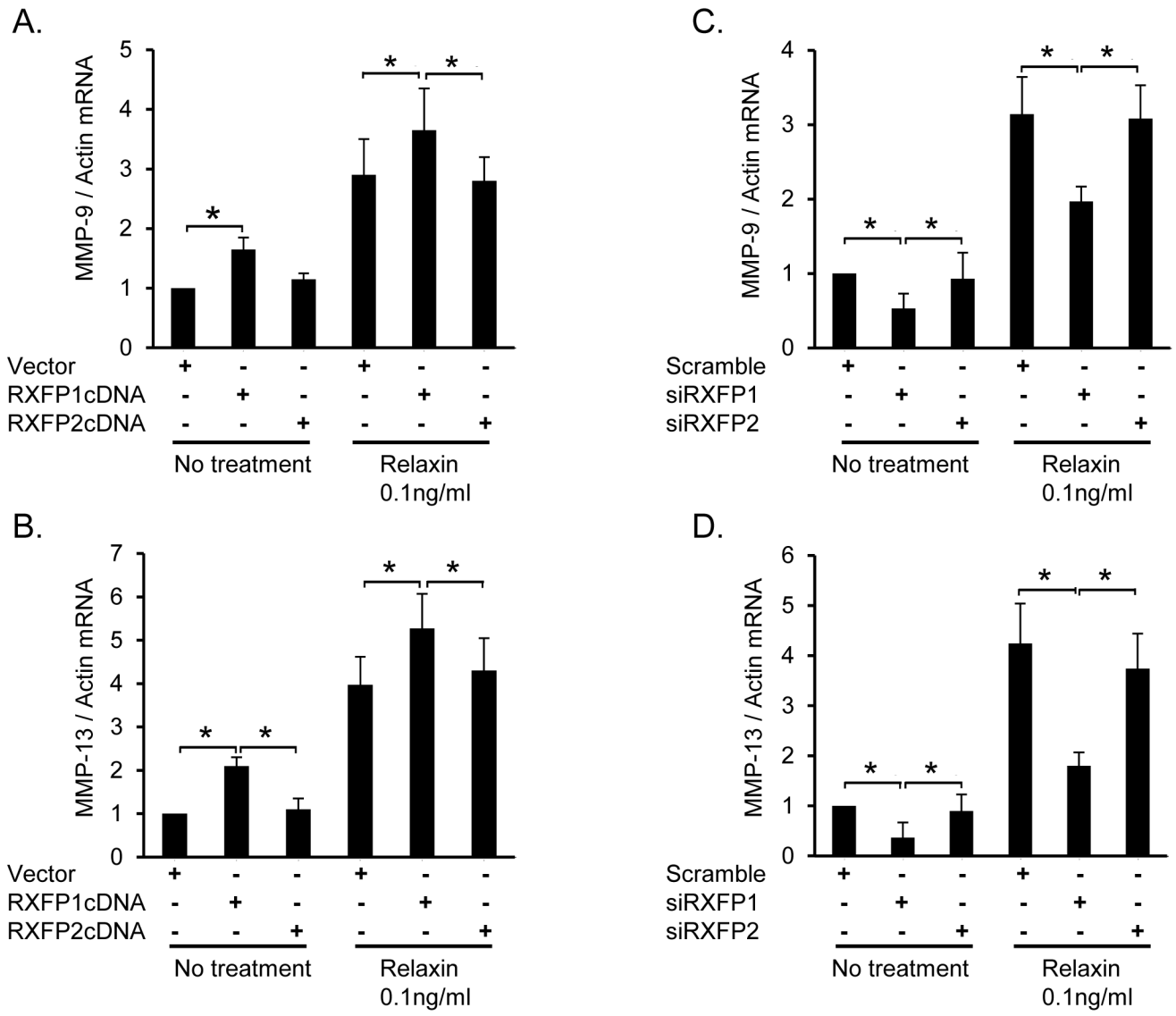

Fig. 5. Relaxin induces MMP -9 and -13 via RXFP1 but not RXFP2

Cells transiently transfected with pcDNA vector or RXFP1 cDNA or RXFP2 cDNA (A and B), or with scrambled siRNA or RXFP1 siRNA or RXFP2 siRNA (C and D), were cultured in the absence or presence of relaxin (0.1ng/ml). Total RNA was isolated, reverse transcribed and subjected to qRT-PCR using MMP-9 (A and C) and MMP-13 (B and D) specific primers. Data is shown as mean (±SE) fold-change in MMP mRNA levels relative to empty vector transfected and untreated cells. (*$p < 0.05$).

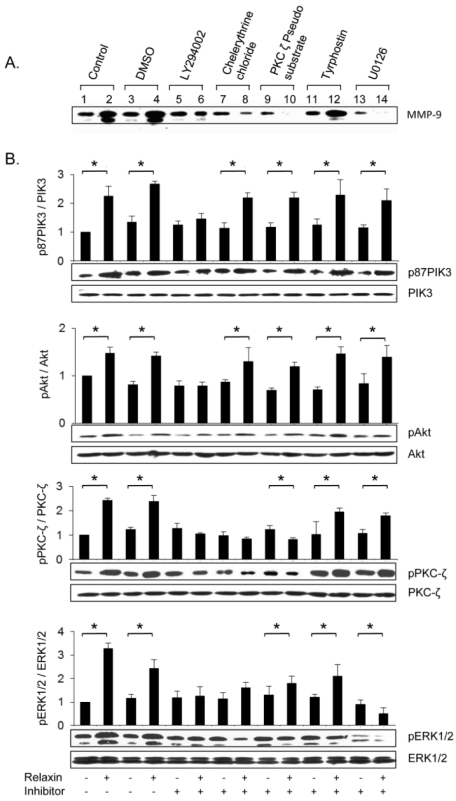

Fig. 6. Activation of RXFP1 by relaxin promotes phosphorylation of signaling molecules PI3K, Akt, PKC-ζ, and ERK1/2 in parallel with induction of MMP-9

Fibrochondrocytes were pre-treated with signaling inhibitors, 2μM LY294002 (PI3K), 1μM chelerythrine chloride (PKC), 2μM PKC-mrystilated pseudosubstrate (PKC-ζ), 1μM tyrphostins (insulin), 1μM U1026 (MEK/ERK) or vehicle (DMSO) for 30 minutes and then stimulated with 0.1ng/ml relaxin. Western blots were performed for MMP-9 in cell-conditioned media collected at 6 hours (A), and for signaling molecules (B) in cell lysates retrieved after 30 minutes. The histograms in B represent mean (± SE) fold change relative to untreated controls of phosphorylated signaling molecule standardized to the respective unphosphorylated protein from Western blots from three independent experiments. Relaxin treatment increased the phosphorylated forms of PI3K, Akt, PKC-ζ and ERK1/2. Relaxin's induction of MMP-9 was inhibited by PI3K, PKC, PKC-ζ and ERK inhibitors. Tyrphostins, which inhibit insulin signaling, served as negative control. Actin was used a loading control. (*$p < 0.05$).

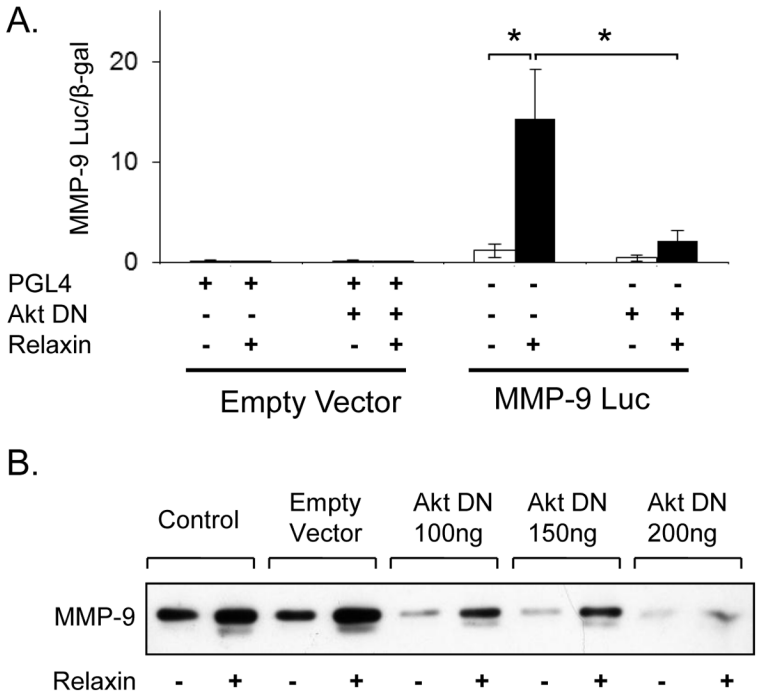

Fig. 7. Relaxin's induction of MMP-9 expression is Akt dependent

Fibrochondrocytes were transiently transfected with empty vector or PGL4 vector or Akt dominant negative construct (Akt DN) or MMP-9 promoter-luciferase construct and cultured in the absence or presence of relaxin (0.1ng/ml). Cell lysates were collected after 6 hours and subjected to luciferase assay. (A) Relaxin treatment increased the expression of MMP-9-promoter linked luciferase activity, which was significantly reduced in cells transfected with Akt DN construct. (B) Conditioned medium from cells transiently transfected with PGL4 vector and increasing concentrations of Akt DN showed induction of MMP-9 induction by relaxin, which was dose-dependently reduced in the presence of Akt DN construct. (*p<0.05)

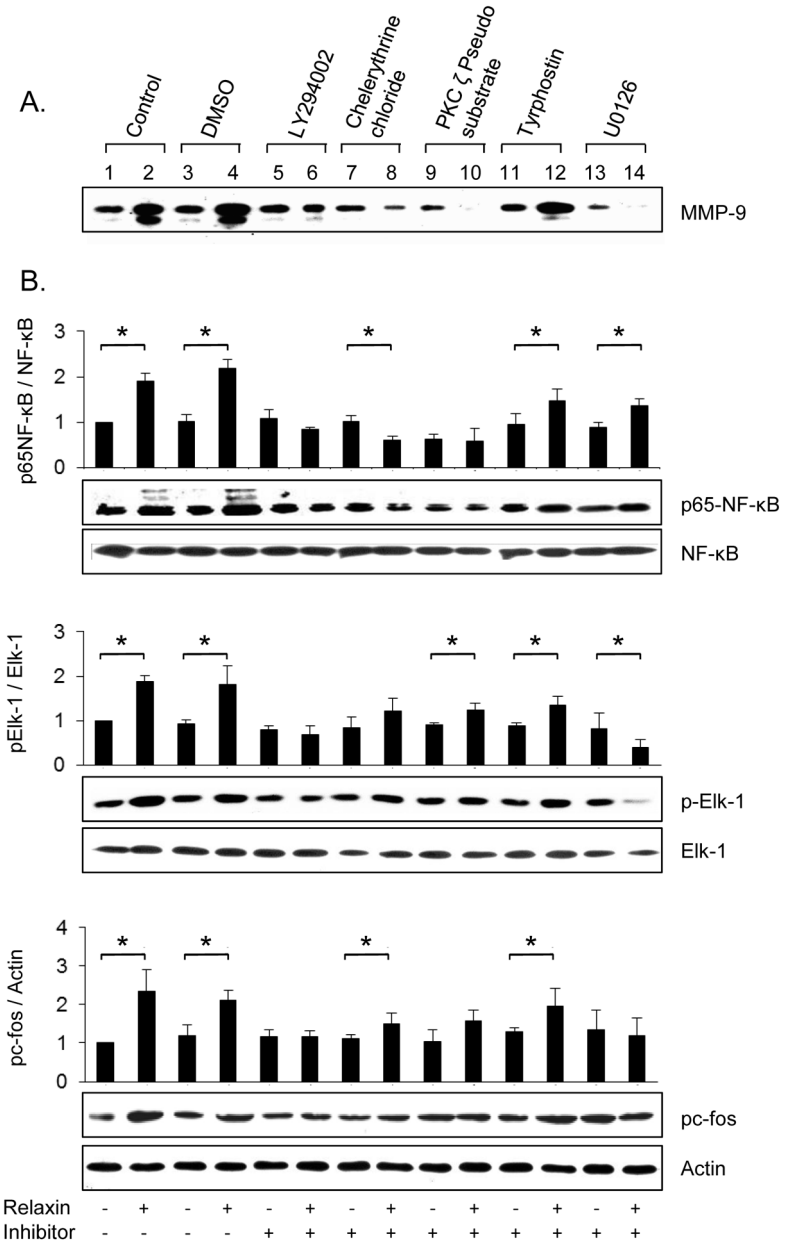

Fig. 8. Activation of RXFP1 by relaxin promotes phosphorylation of transcription factors NF-κB, Elk-1 and c-fos downstream of PI3K, PKC, PKC-ζ and ERK1/2 in parallel with induction of MMP-9

Fibrochondrocytes were pre-treated with signaling inhibitors or vehicle (DMSO) for 30 minutes and then stimulated with 0.1ng/ml relaxin as described in Figure 6. Western blots were performed for MMP-9 in cell-conditioned media collected at 6 hours (A), and transcription factors (B) in cell lysates retrieved after 30 minutes. The histograms in B represent mean (± SE) fold change relative to untreated controls of phosphorylated transcription factors standardized to the respective unphosphorylated proteins from Western blots from three independent experiments. Relaxin treatment increased the phosphorylated forms of NF-κB, Elk-1 and c-fos. Relaxin's induction of MMP-9 was inhibited by PI3K,

PKC, PKC-ζ and ERK inhibitors. Tyrphostins, which inhibit insulin signaling, served as negative control.. Actin was used a loading control. (*$p < 0.05$).

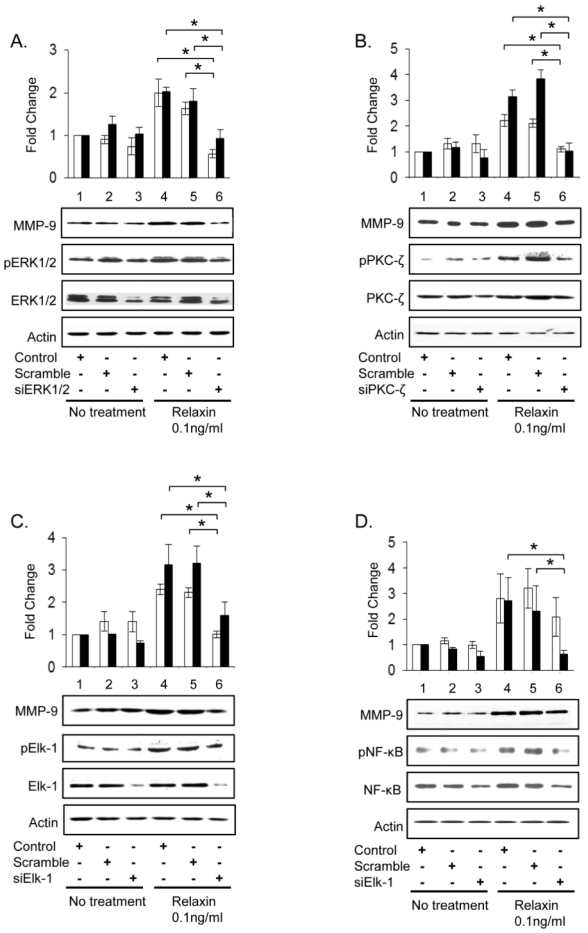

Fig. 9. Silencing of ERK1/2, PKC-ζ, Elk-1 eliminates relaxin's induction of MMP-9

Untransfected cells (Control) or cells transiently transfected with scrambled siRNA, or siRNA to ERK1/2, PKC-ζ, Elk-1 or NF-κB were cultured in the absence or presence of relaxin (0.1ng/ml). Western blots for ERK1/2 (A), PKC-ζ (B), Elk-1 (C) and NF-κB (D) demonstrate successful silencing of the respective molecules and their phosphorylated forms by siRNA transfections. Western blots for MMP-9 show that suppression of ERK1/2, PKC-ζ, or Elk-1 results in elimination of relaxin's induction of MMP-9 (A to C). (D) NF-κB suppression results in minimal inhibition of induction of MMP-9 by relaxin. The histograms represent mean (± SE) fold change of MMP-9 (white bars) and phosphorylated (black bars) proteins from Western blots from three independent experiments. Actin was used as an internal control. (*$p < 0.05$).

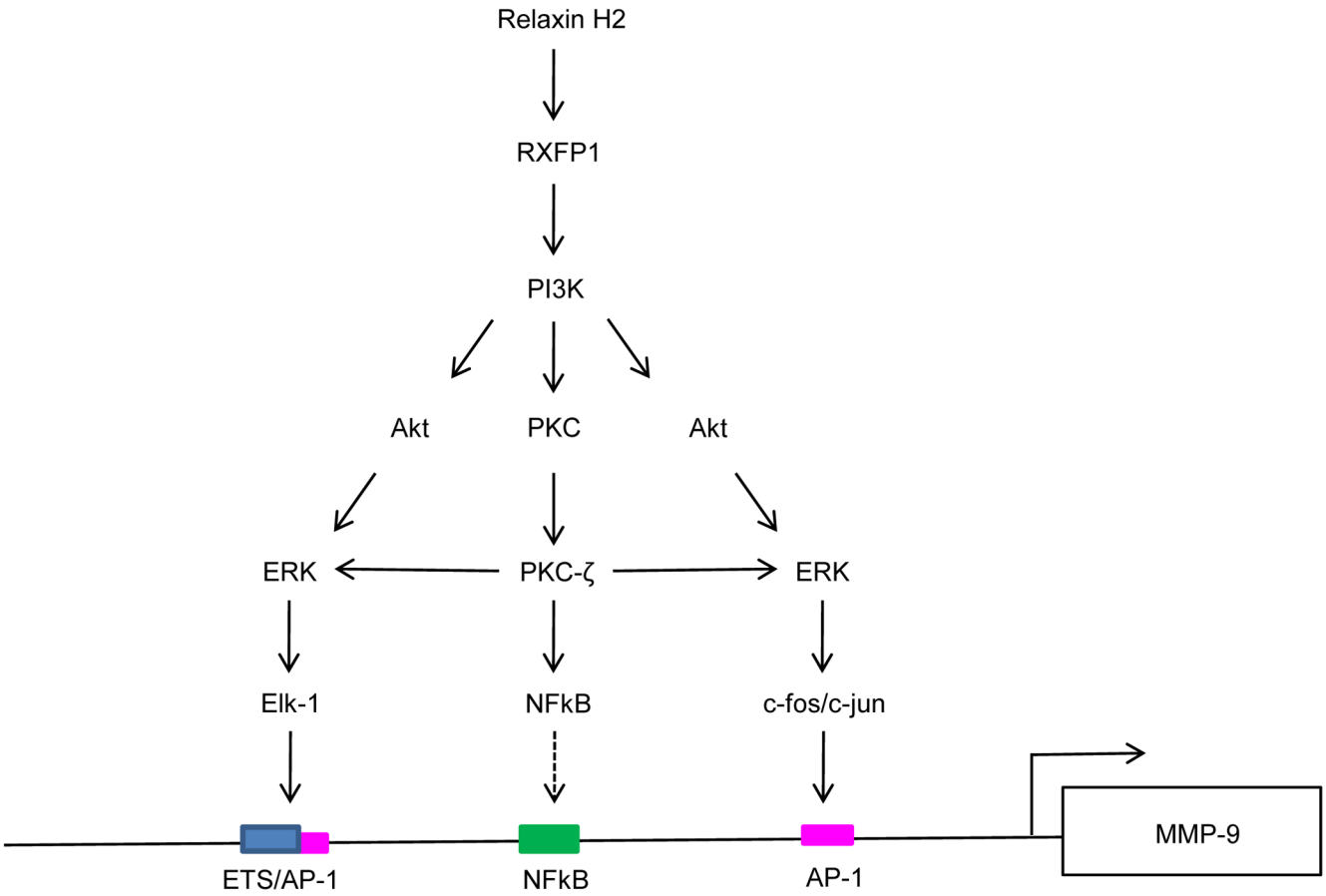

Fig. 10. Schematic representation of signaling pathways involved in relaxin's induction of MMP-9 in fibrochondrocytes

Relaxin binds to RXFP1 receptor and activates PI3K/Akt, ERK and PKC-ζ signaling pathways, which leads to the phosphorylation of DNA binding transcriptional factors Elk-1, c-fos and NF-κB. Silencing of Elk-1 and c-fos and to a lesser extent NF-κB eliminated relaxin-induced MMP-9 expression in fibrochondrocytes.